Hoxc6 Is Overexpressed in Gastrointestinal Carcinoids and Interacts With JunD to Regulate Tumor Growth

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Background & Aims: **The molecular alterations that underlie carcinoid tumor pathogenesis remain poorly defined. The homeobox gene *HOXC6* was highly up-regulated in human gastrointestinal carcinoid tumors, and we sought to define its pathogenic role.** ***Methods:*** **The functional and physical properties of Hoxc6 were investigated by establishing carcinoid cells that stably overexpressed Hoxc6 or were deficient in Hoxc6. Cellular proliferation assays, luciferase reporter assays, Western blotting, immunoprecipitation, DNA affinity precipitation, and DNA microarray studies were performed.** ***Results:*** **Expression of Hoxc6 in cultured human BON1 carcinoid cells enhanced their proliferation, and knock-down of Hoxc6 inhibited their growth. Hoxc6 activated the oncogenic activator protein-1 signaling pathway through a physical interaction with JunD. Mutations in the homeodomain of Hoxc6 blocked this interaction and inhibited proliferation of carcinoid cells. Of note, Hoxc6 induced the expression of genes that characteristically are up-regulated in carcinoid tumors, including neurotensin and connective tissue growth factor.** ***Conclusions:*** **A novel molecular pathway has been identified that links Hoxc6 with oncogenic signaling through the activator protein-1 pathway in carcinoid tumorigenesis.**

Carcinoid tumors are neuroendocrine neoplasms that can arise in multiple organ sites, but most commonly are localized to the gastrointestinal tract.[1] They typically are slow-growing, well-differentiated tumors that secrete 5-hydroxytryptamine, and excess levels can result in the clinical carcinoid syndrome.[2] Gastrointestinal carcinoids often are classified together with neuroendocrine tumors of the pancreas because they share many clinical and biological features. However, key insights into their underlying molecular pathogenesis are lacking.

Individuals with the multiple endocrine neoplasia type 1 (MEN1) syndrome are at high risk for the development of neuroendocrine tumors of the pancreas and pituitary, as well as parathyroid gland hyperplasia and gastric carcinoids. The *MEN1* gene that underlies the syndrome was cloned in 1997.[3] *MEN1* also plays a role in sporadic pancreatic neuroendocrine tumors and gastrointestinal carcinoids, but only a subset of these harbors *MEN1* mutations.[4] The *MEN1* gene product, menin, has diverse functions,[5] and most recently has been shown to regulate gene transcription through interactions with a histone methyltransferase complex.[6,7] Some of the targets of menin include the homeobox genes *HOXC6* and *HOXC8*,[6] as well as the cell-cycle inhibitors p18 and p27.[8,9]

Hox genes belong to the homeoprotein family of transcription factors that are developmental regulators of growth, patterning, and differentiation. There are 4 clusters of Hox genes (*HOXA*, *HOXB*, *HOXC*, and *HOXD*), and Hox proteins contain a homeodomain that consists of 3 helices separated by a short loop. Helix III (the C-terminal helix) contacts A/T-rich domains of DNA in the regulatory regions of many genes.[10] Because most homeobox proteins have similar DNA binding specificity in vitro, the timing of expression as well as the interactions between Hox proteins and other transcriptional regulators are likely to be critical for their functional specificity. Deregulated expression of Hox genes has been reported in many tumors, including lung, breast, ovarian, sarcoma, and leukemia.[10] The specific mechanisms by which Hox genes contribute to the tumorigenic phenotype are incompletely described. In some cases, they may function as transcription factors that stimulate the expression of growth factors, such as Hoxb7-mediated up-regulation of fibroblast growth factor in melanomas.[11] Overexpression of Hoxc6 is observed in human prostate cancer, where it functions to inhibit apoptosis.[12,13]

Because of the link between the *HOXC6* and *HOXC8* gene clusters and the *MEN1* gene,[6] we were curious whether expression of these Hox genes was altered in human neuroendocrine tumors. Previous DNA microarray studies have indicated that Hoxc6 messenger RNA

Abbreviations used in this paper: AP-1, activator protein-1; CT, threshold cycle; CTGF, connective tissue growth factor; Hoxc6-V1, homeobox c6 variant 1; Hoxc6-V2, homeobox c6 variant 2; MEN1, multiple endocrine neoplasia 1; mHoxc6, mutant homeobox c6; NT, neurotensin; RT-PCR, reverse-transcription polymerase chain reaction; siRNA, small interfering RNA.

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Table 1. Clinical Features of Neuroendocrine Tumor Samples

Sample	Age, y	Sex	Primary site	Tumor size, cm	Stage	Carcinoid syndrome
Gastrointestinal carcinoids						
2974-1	72	NK	Ileum	0.4	Metastatic to liver	Yes
11898-1	52	F	Ileum	2	Metastatic to liver	Yes
33762-1	67	M	Ileum	1.5	Metastatic to liver	Yes
53456-1	59	F	Colon	NK	Metastatic to liver	NK
67494-1	53	F	Ileum	2.5	T3N1M0	No
80670-1	70	F	Ileum	3.5	Metastatic to liver	No
139	55	F	Ileum	1.7	Metastatic to liver	No
177	51	F	Ileum	2	Metastatic to liver	Yes
183	58	M	Small bowel	1.6	Metastatic to liver	Yes
192	56	F	Small bowel	3	Metastatic to liver	No
205	57	M	Ileum	3.1	Metastatic to liver	Yes
	Age, y	Sex	Tumor type	Tumor size, cm	WHO classification	Metastases
Pancreatic neuroendocrine tumors						
2	62	M	PTHrPoma	8.5	WDEC	Lymph nodes, liver
53	69	F	Nonfunctional	13	WDET-LM	None
56	73	M	Insulinoma	2.5	WDEC	Lymph nodes
59	65	M	ACTHoma	6.5	WDEC	Lymph nodes, liver
61	58	F	Gastrinoma	12	WDEC	Duodenal wall invasion
69	48	F	Gastrinoma	3	WDEC	Lymph nodes
83	47	F	Nonfunctional	7	WDEC	Lymph nodes
84	59	M	Gastrinoma	NK	WDEC	Lymph nodes

NK, not known; WDEC, well-differentiated endocrine carcinoma; WDET-LM, well-differentiated endocrine tumor, low-grade malignant; PTHrPoma, parathyroid hormone-related protein secreting tumor; ACTHoma, adrenocorticotropic hormone secreting tumor.

(mRNA) was strongly up-regulated in gastrointestinal carcinoid tumors but not in closely related pancreatic neuroendocrine tumors.[14] There are 2 Hoxc6 variants that arise from differential promoter use.[15] Hoxc6-variant 1 (Hoxc6-V1) is derived from a 1.8-kb mRNA transcript encoding a 27-kilodalton protein, and Hoxc6-variant 2 (Hoxc6-V2) is derived from a 2.2-kb mRNA transcript that encodes an 18-kilodalton protein. Although both possess the identical 61 amino acid homeodomain, their functional differences are unknown. We now verify that Hoxc6 is overexpressed in human carcinoid tumors and show that expression of Hoxc6 stimulated the growth of carcinoid cells in vitro. Hoxc6 enhanced signaling through the activator protein-1 (AP-1) pathway, and this was mediated by a novel interaction between Hoxc6 and the JunD component of AP-1. These findings link a developmental regulator to an oncogenic signaling pathway. In addition to providing an important new insight into the pathogenesis of carcinoid tumors, these studies point to a molecular pathway that may be a novel target for therapy in carcinoids.

Materials and Methods

Quantitative Polymerase Chain Reaction and Reverse-Transcription Polymerase Chain Reaction

RNA from 11 carcinoid tumors (Table 1), 8 pancreatic endocrine tumors, 2 samples of normal ileum, and 6 samples of normal pancreas were isolated using the RNeasy Protect Mini Kit (QIAGEN, Valencia, CA). Reverse transcription (RT) was performed using the SuperScript III platinum Two-Step qRT-polymerase chain reaction (PCR) Kit (Invitrogen, Carlsbad, CA). The primers for Hoxc6-V1 were as follows: sense 5'- CCTTCCTTATCCTGCCACCT-3', antisense 5'-GCTGGAACTGAACACGACAT-3' (NM_004503). The primers for Hoxc6-V2 were as follows: sense 5'-CTCCTCTCTCCCAGGCTCTT-3', antisense 5'-GCTGCTGTCACTCTCCCTCT-3' (NM_153693). The primers for Hoxc8 were as follows: sense 5'-CTCAGGCTACCAGCAGAACC-3', antisense 5'-TTGGCGGAGGATTTACAGTC-3' (NM_022658). 18S ribosomal RNA served as an endogenous control. PCR cycles were as follows: 2 minutes at 95°C, followed by 40 cycles with an annealing temperature of 60°C. A fluorogenic SYBR Green and MJ Research (Waltham, MA) detection system were used for real-time quantification. Primer sequences for neurotensin (NT), peptide YY, and connective tissue growth factor (CTGF) are available on request.

The results are presented as parameter threshold cycle (CT) values. ΔCT is the difference in the CT values derived from the specific gene being assayed and 18S ribosomal RNA whereas ΔΔCT represents the difference between the paired samples, as calculated by the formula ΔΔCT = ΔCT of a sample -ΔCT of a reference. The amount of target, normalized to 18S and relative to a reference, was expressed as $2^{-\Delta\Delta CT}$.

Stable Cell Lines

BON1 cells (a human pancreatic carcinoid cell line) were used to generate cells stably overexpressing

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